Clinical Review

HPV Infection of the Head and Neck Region and Its Stem Cells

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Abstract

The human papillomavirus (HPV) is an etiologic agent associated with the development of head and neck squamous carcinoma (HNSCC)—in particular, oropharyngeal squamous cell carcinoma. The HPV-positive HNSCC is characterized by genetic alterations, clinical progression, and therapeutic response, which are distinct from HPV-negative head and neck cancers, suggesting that virus-associated tumors constitute a unique entity among head and neck cancers. Malignant stem cells, or cancer stem cells, are a subpopulation of tumor cells that self-renew, initiate new tumors upon transplantation, and are resistant to therapy, and their discovery has revealed novel effects of oncovirus infection in cancer. In this review, we provide a virus-centric view and novel insights into HPV-positive head and neck pathogenesis. We discuss the influence of cancer stem cells, HPV oncoproteins, altered molecular pathways, and mutations in cancer initiation and cancer progression. We compiled a catalogue of the mutations associated with HPV-positive HNSCC, which may be a useful resource for genomic-based studies aiming to develop personalized therapies. We also explain recent changes in mass vaccination campaigns against HPV and the potential long-term impact of vaccinations on the prevention and treatment of HPV-positive head and neck cancers.

Keywords: head and neck cancer, oral cancer, mutation, cancer stem cells, molecular sequence data, HPV vaccine

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth-most common cancer worldwide, with an annual incidence of approximately 600,000 cases (Ferlay et al. 2010; Blitzer et al. 2014). While HNSCC has traditionally been connected with tobacco and alcohol exposure, an increasing number of HNSCC cases have been attributed to human papillomavirus (HPV) infection in the past decade (Chaturvedi et al. 2011; Blitzer et al. 2014). HNSCC is caused by noninfectious factors, infectious agents (Parkin and Bray 2006), and inherited predisposition factors, such as the inherited chromosomal instability disorders, Fanconi anemia, and Bloom syndromes (Kutler et al. 2003; Barnes et al. 2005).

HPV infection is an emerging risk factor for HNSCC development in the oropharynx. Moreover, the incidence of HPV-positive HNSCC is increasing at an alarming rate in the United States (Ang et al. 2010; Chaturvedi et al. 2011; Gillison et al. 2012; Akagi et al. 2014; Blitzer et al. 2014). HPV-associated HNSCC is expected to become the primary cause of HNSCC in the United States and other countries that are experiencing a decrease in tobacco use (Chaturvedi et al. 2011; Blitzer et al. 2014). Recent studies aimed at better understanding HPV-associated oropharyngeal pathology revealed that HPV-positive tumors have distinct epidemiologic and prognostic characteristics. HPV-positive oropharyngeal cancer has a better response to therapy and improved prognosis than do HPV-negative tumors (Gillison et al. 2012). These data support recognizing HPV-positive oropharyngeal HNSCC as a distinct cancer from HPV-negative tobacco-related HNSCC (Lassen 2010).

As early as the mid-19th century, sexual activity was suspected to be a risk factor for cancer, particularly cervical cancer. Initial experiments conducted by Francis Rous, the 1966 Nobel laureate, explored the ability of viruses to transmit and/or initiate cancer (Moore and Chang 2010). Research efforts in the 20th century resulted in the identification of important factors associated with cellular transformation and tumor progression. After the landmark discovery of the Epstein-Barr virus as the infectious agent responsible for Burkitt lymphoma, other viruses were found to cause cancer, including HPV, which is now associated with oropharyngeal cancer development and progression.

Over 160 genotypes or strains of HPV have been identified, including the selective high-risk oncogenic subtypes belonging to the α-HPV genus. With approximately 15 high-risk subtypes, HPV16 and HPV18 remain the major cause of

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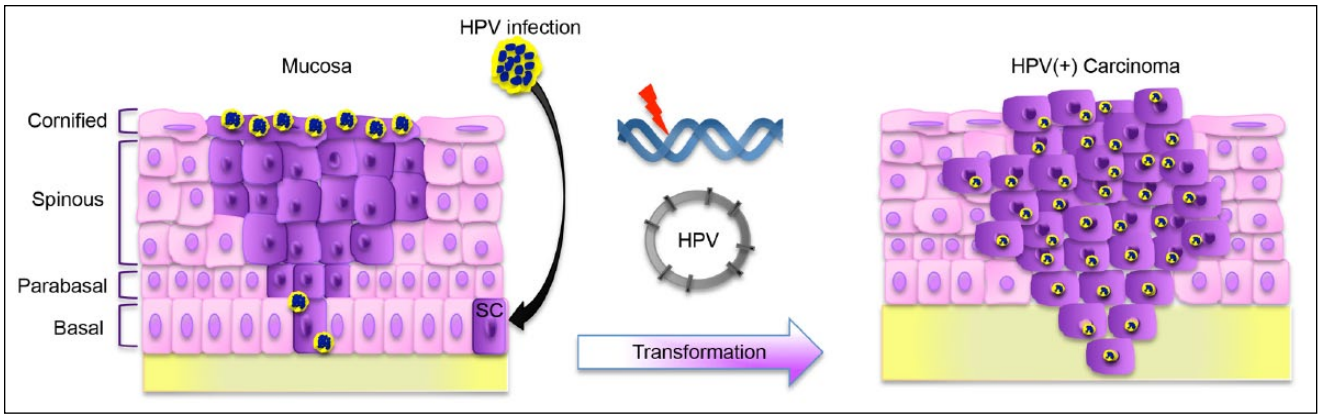

Figure 1. Human papillomavirus (HPV) infection and acquired mutations promote cell transformation and head and tumor progression. The diagram depicts HPV preferably infecting stem cells and epithelial cells located at the basal layer of the oral mucosa. The infected cells give rise to a group of differentiating cells that incorporate DNA damage and mutations that in time promote cellular immortalization, transformation, and development of HPV-associated head and neck cancer. Note that HPV alone is not sufficient to transform epithelial cells, even though they are greatly used in the maintenance of cancer. Secondary genetic and epigenetic events to HPV infection are necessary for cellular transformation and head and neck cancer development and progression.

HPV-associated cancers (Moody and Laimins 2010; Moore and Chang 2010). The high-risk HPV16 and HPV18 strains were discovered by Harald zur Hausen and colleagues (Dürst et al. 1983), and their work later culminated in the 2008 Nobel Prize in Physiology or Medicine. HPV16 causes a 50-fold increased risk for HPV-positive HNSCC and is present in 50% to 90% of HPV-positive HNSCC (Ang et al. 2010; Chaturvedi et al. 2011; Gillison et al. 2012; Akagi et al. 2014; Blitzer et al. 2014), while other HPV types, such as HPV31/33/58/59/62/72, are less common (Koskinen et al. 2003).

From Epithelial Infection to Tumor Initiation: Viral Integration, Gene Mutations, and Chromosomal Rearrangements

High-risk HPV is a direct carcinogen and may gain entry via microabrasions or trauma to the oropharynx mucosa as well as to genital mucosa and skin. HPV displays tropism toward the epithelial basal layer, which houses adult epithelial stem cells responsible for replenishing the epithelium with daughter cells as shown in Figure 1 (Clayton et al. 2007; Letian and Tianyu 2010; Hsu and Fuchs 2014; Egawa et al. 2015). HPV infects epithelial cells via interaction with cell surface receptors such as integrin α6 (Letian and Tianyu 2010), which is abundant in basal cells and epithelial stem cells (Kaur and Li 2000; Hsu and Fuchs 2014). In infected cells and premalignant lesions, the majority of the HPV genome remains in the episomal state. In contrast, HPV integrates into the host genome in malignant tumors; therefore, integration of HPV into the genome may be the primary evidence of cancer (Feng et al. 2008). HPV integrants are found in multiple nonrecurrent regions of amplification and in flanked regions where deletions occur (Akagi et al. 2014; Cancer Genome Atlas Network 2015). There is a strong association between HPV insertional breakpoints and genomic structural variations, including chromosomal translocations, deletions, inversions, and intrachromosomal rearrangements, ultimately resulting in genomic instability, a hallmark of HPV-positive cancers (Akagi et al. 2014).

The HPV double-stranded DNA genome in the host cells encodes 8 viral proteins: L1, L2, E1, E2, E4, E5, E6, and E7. The 3 major viral oncoproteins (E5, E6, and E7) contribute to cancer initiation and progression by altering cell cycle regulation and telomere maintenance, inducing DNA damage and genomic instability, and blocking tumor suppressor pathways and apoptosis (Moody and Laimins 2010). The E6/E7 oncoproteins are the primary transforming viral proteins, and they play an important role in immune evasion by targeting cytokine expression to alter cell proliferation and interferon responses. E7 proteins modulate genome-wide transcription through their interactions with histone deacetylases, which activate transcription when removed from promoters (Longworth and Laimins 2004).

Importantly, expression of HPV viral proteins and viral integration promotes chromosomal anomalies and cellular immortalization. Nevertheless, HPV alone is not sufficient to transform epithelial cells, although it plays a significant role in maintaining cancer. Genetic and epigenetic events that are secondary to HPV infection are necessary for cellular transformation and cancer development (Moody and Laimins 2010; Akagi et al. 2014). This phenomenon may explain, in part, the latency period that occurs in cancer development. Mutations that may contribute to HPV-positive HNSCC are described in Table 1. Future studies that explore the mutations and cooperative actions between HPV and epigenetic and genetic events are essential to better understanding HPV-associated HNSCC development and progression. Furthermore, studies on mutations associated with HPV-positive HNSCC may be helpful resources for genomic-based studies aiming at the identification of molecular markers associated with HPV infection and development of personalized therapies. Future vaccination

Table 1. Mutations in Human Papillomavirus–positive Head and Neck Squamous Cell Carcinoma.

Protein				Gene				
Name	Alias	UniProt	Amino Acid Change	Name	Alias	Tobacco Use	Alcohol Cons	Reference
ABL1	p150, c-Abl	P00519	p.N350D	*ABL1*	*ABL, JTK7*	Yes	Occas	Lechner et al.
ABL2	Tyrosine-protein kinase ARG	P42684	p.T769S	*ABL2*	*ABLL, ARG*	Yes	Occas	Lechner et al.
AML1	CBF-alpha-2, PEA2-alpha B, PEBP2-alpha B	Q01196	p.R250H	*RUNX1*	*AML1, CBFA2*	Unkn	No	Lechner et al.
APC	Deleted in polyposis 2.5	P25054	p.S2544L	*APC*	*DP2.5*	Yes	Occas	Lechner et al.
Apo B-100	Apo B-48; Apolipoprotein B-100	P04114	p.N1080S	*APOB*	N/A	No	Unkn	Agrawal et al.
ANDR	Dihydrotestosterone receptor	P10275	p.N849fs*32	*AR*	*DHTR, NR3C4*	No	No	Lechner et al.
ARID1A	BAF250, B120; BAF250A, hOSA1, SWI-like protein; hELD	O14497	p.E1779G	*ARID1A*	*BAF250, BAF250A, C1orf4, OSA1, SMARCF1*	Unkn	No	Lechner et al.
ATM	A-T mutated	Q13315	p.R2161H	*ATM*	N/A	No	>24	Lechner et al.
			p.N1356D			Yes	>24	
BCoR-L1	BCoR-L1; BCoR-like protein 1	Q5H9F3	p.R784X	*BCORL1*	N/A	Yes	Unkn	Agrawal et al.
B-Raf	p94, v-Raf	P15056	p.E24D	*BRAF*	*BRAF1, RAFB1*	Yes	Occas	Lechner et al.
BRCA1	RING finger protein 53	P38398	p.L246V	*BRCA1*	*RNF53*	No	Occas	Lechner et al.
			p.A622V			Yes	Occas	
BRCA2	Fanconi anemia group D1 protein	P51587	p.S326R	*BRCA2*	*FACD, FANCD1*	No	No	Lechner et al.
			p.L709V			Yes	Occas	
BRG1	BAF190A, SMARCA4, SNF2-beta	P51532	p.R359Q	*SMARCA4*	*BAF190A, BRG1, SNF2B, SNF2L4*	No	No	Lechner et al.
Cadherin-2	N-cadherin, CDw325, CD325	P19022	p.G473E	*CDH2*	*CDHN, NCAD*	Yes	Occas	Lechner et al.
Cadherin-5	VE-cadherin; 7B4 antigen; CD144	P33151	p.K768Q	*CDH5*	N/A	Unkn	No	Lechner et al.
			p.V359I			Yes	Occas	
CDKI2A	CDK4I, MTS-1, p16-INK4, p16INK4A, p14ARF, p19ARF	P42771	p.N71D	*CDKN2A*	*CDKN2, MTS1*	No	Unkn	Fischer; Agrawal et al.
			p.H98R			Unkn	Unkn	
			p.A57P			Unkn	Unkn	
			p.D84fs*51			Unkn	Unkn	
			p.E69fs*51			Unkn	Unkn	
			p.R107fs*13			Unkn	Unkn	
			p.A57P			Unkn	Unkn	
			p.A60V			Unkn	Unkn	
DNA-PKcs	DNPK1, p460	P78527	p.S1512N	*PRKDC*	*HYRC, HYRC1*	Yes	Occas	Lechner et al.
			p.V1434I			Yes	Occas	
			p.R1735Q			Yes	>24	
			p.N3605del			Yes	Occas	
Dystonin	BPA	Q03001	p.Q1301X	*DST*	*BP230, BP240, BPAG1, DMH, DT, KIAA0728*	No	Unkn	Agrawal et al.
E-NPP 1	PC-1	P22413	p.A494D	*ENPP1*	*M6S1, NPPS, PC1, PDNP1*	No	Unkn	Agrawal et al.
EPH receptor A3	EK4, hEK4, TYRO4, HEK, ETK1	P29320	p.L10F	*EPHA3*	*ETK, ETK1, HEK, TYRO4*	Yes	>24	Lechner et al.
EPH receptor A7	EHK-3, EK11, hEK11	Q15375	p.E712Q	*EPHA7*	*EHK3, HEK11*	Yes	Occas	Lechner et al.
			p.Y399*			Yes	Occas	Lechner et al.
EPH receptor B3	EK2, EPH-like kinase 2, hEK2, TYRO6	P54753	p.V701F	*EPHB3*	*ETK2, HEK2, TYRO6*	No	Unkn	Agrawal et al.
EPH receptor B6	HEP, EPH-6	O15197	p.V737M	*EPHB6*	N/A	No	No	Lechner et al.
			p.E867K			Yes	Occas	
			p.G182E			Yes	Occas	
erbB-2	MLN 19, Neu, c-ErbB-2, HER2, p185erbB2, CD340	P04626	p.E1114K	*ERBB2*	*HER2, MLN19, NEU, NGL*	Yes	Occas	Lechner et al.
ERG	Transforming protein ERG	P11308	p.P116R	*ERG*	N/A	Yes	Occas	Lechner et al.
ER	ER-alpha	P03372	p.K299R	*ESR1*	*ESR, NR3A1*	Yes	Occas	Lechner et al.

(continued)

Table 1. (continued)

Protein				Gene				
Name	Alias	UniProt	Amino Acid Change	Name	Alias	Tobacco Use	Alcohol Cons	Reference
Erythrocytic 1	Erythroid alpha-spectrin	P02549	p.R1224W	*SPTA1*	*SPTA*	Yes	Unkn	Agrawal et al.
FANCA	N/A	O15360	p.H292D	*FANCA*	*FAA, FACA, FANCH*	No	No	Lechner et al.
FBXW7	hAgo, FBX30, SEL-10, hCdc4	Q969H0	p.R505H	*FBXW7*	*FBW7, FBX30, SEL10, CDC4*	No	No	Wang et al.; Lechner et al.; Agrawal et al.
			p.R505C			No	No	
			p.R505G			Unkn	Unkn	
			p.R505L			Yes	Unkn	
			p.R479Q			No	No	
			p.R465H			Unkn	Unkn	
			p.R367*			Yes	Occas	
FGFR-2	KGFR, CD332	P21802	p.K659N	*FGFR2*	*BEK, KGFR, KSAM*	No	No	Lechner et al.
FGFR-4	CD334	P22455	p.V10G	*FGFR4*	*JTK2, TKF, CD334*	Yes	Occas	Lechner et al.
GH-V	hGH-V	P01242	p.G209R	*GH2*	N/A	Yes	Unkn	Agrawal et al.
			p.N125K			Yes	Unkn	
GNAS	ALEX, NESP55	P84996	p.Q96E	*GNAS*	*GNAS1, GNAS* complex locus	Yes	Occas	Lechner et al.
GPCR124	Tumor endothelial marker 5	Q96PE1	p.E150K	*GPR124*	*KIAA153, TEM5*	No	>24	Lechner et al.
GTPase Kras	K-Ras, K-Ras 2, Ki-Ras, c-K-ras, c-Ki-ras	P01116	p.G12D	*KRAS*	*KRAS2, RASK2*	Yes	Occas	Lechner et al.
Hamartin	TSC1	Q92574	p.H732Y	*TSC1*	*TSC, KIAA0243*	Yes	Occas	Lechner et al.
HGF receptor	c-Met, SF receptor, HGF/SF receptor, Met	P08581	p.V13M	*MET*	N/A	No	No	Lechner et al.
			p.D1117N			No	>24	
			p.R1004*			Yes	Occas	
hMSH2	MutS protein homolog 2, MSH2	P43246	p.I633T	*MSH2*	N/A	Yes	>24	Lechner et al.
hPER2	Circadian clock protein PERIOD 2	O15055	p.Y1102X	*PER2*	*KIAA0347*	Yes	Unkn	Agrawal et al.
hPLD2	PLD1C, PLD 2, Choline phosphatase 2	O14939	p.E851K	*PLD2*	N/A	Yes	Unkn	Agrawal et al.
HSP 90-alpha	HSP 86, HS 86, LAP-2, NY-REN-38, LPS-associated protein 2	P07900	p.K396del	*HSP90AA1*	*HSP90A, HSPC1, HSPCA*	Unkn	No	Lechner et al.
			p.S10A			Yes	Occas	
IGF-1	CD221	P08069	p.P190S	*IGF1R*	N/A	No	No	Lechner et al.
JAK1	JAK-1	P23458	p.S383G	*JAK1*	*JAK1A, JAK1B*	Yes	Occas	Lechner et al.
Leucine-rich repeat neuronal 6C	Leucine-rich repeat neuronal protein 3 or 6C	Q7L985	p.D492N	*LINGO2*	*LERN3, LRRN6C*	Yes	Unkn	Agrawal et al.
LRP-1B	LRP-DIT	Q9NZR2	p.N2501D	*LRP1B*	*LRPDIT*	Yes	Occas	Lechner et al.
Lysine N-methyl-transferase 2A	ALL-1, MML, MML1, HRX, TRX1	Q03164	p.G3097fs*2	*MLL*	*ALL1, CXXC7, HRX, HTRX, KMT2A, MLL1, TRX1*	Yes	Occas	Lechner et al.
MAPKK 2	MEK 2	P36507	p.P329S	*MAP2K2*	*MEK2, MKK2, PRKMK2*	Yes	>24	Lechner et al.
MICAL2	MICAL-2	O94851	p.R1024W	*MICAL2*	*KIAA0750, MICAL2PV1, MICAL2PV2*	No	Unkn	Agrawal et al.
MITF	bHLHe32	O75030	p.T444R	*MITF*	*BHLHE32*	Yes	Occas	Lechner et al.
M6PR	CI Man-6-P receptor, CI-MPR, MPR 300, IGF-II receptor, M6P/IGF2R, CD222	P11717	p.E508fs*48	*IGF2R*	*MPRI*	Unkn	Occas	Lechner et al.
Neurexin-3-beta	Neurexin III-beta	Q9HDB5	p.G368R	*NRXN3*	*KIAA0743*	No	Unkn	Agrawal et al.
NLRP12	Monarch-1	P59046	p.R329Q	*NLRP12*	*NALP12, PYPAF7, RNO*	Yes	Unkn	Agrawal et al.
N-myc	bHLHe37	P04198	p.E185K	*MYCN*	*BHLHE37, NMYC*	Yes	Occas	Lechner et al.
Notch 1	hN, TAN-1	P46531	p.R1438H	*NOTCH1*	*TAN1*	Unkn	Occas	Lechner et al.; Agrawal et al.
			p.C1133F			No	>24	
			p.R353H			Yes	Unkn	
			p.M2011R			Yes	Unkn	
			p.F1292L			No	Unkn	

(continued)

Table 1. (continued)

Protein				Gene				
Name	Alias	UniProt	Amino Acid Change	Name	Alias	Tobacco Use	Alcohol Cons	Reference
NRAS	HRAS1	P01111	p.Q61K	*NRAS*	NS6, CMNS, NCMS, ALPS4, N-ras, NRAS1	Unkn	Unkn	Wang et al.
P53	NY-CO-13	P04637	p.Y205C	*TP53*	*P53*	Unkn	Unkn	Westra et al.
			p.G244S			Unkn	Unkn	Westra et al.
			p.P278T			Unkn	Unkn	Westra et al.
			p.R290C			No	No	Lechner et al.
			p.Q144*			Yes	Unkn	Scholes et al.
			p.K181*			Unkn	Unkn	Scholes et al.
			del187-188 (exon 6)			Unkn	Unkn	Sisk et al.
			p.H193D			Unkn	Unkn	Sisk et al.
			p.N273H			No	Occas	Sisk et al.
PAK3	PAK-3, Beta-PAK, Oligophrenin-3	O75914	p.D175E	*PAK3*	*OPHN3*	Yes	Occas	Lechner et al.
PARP-10	ARTD10	Q53GL7	p.P381S	*PARP10*	N/A	Yes	Unkn	Agrawal et al.
PDGF-R-beta	PDGFR-beta, PDGFR-1, CD140b	P09619	p.T294A	*PDGFRB*	*PDGFR, PDGFR1*	No	No	Lechner et al.
PHLPP2	PHLPP-like	Q6ZVD8	p.S1194F	*PHLPP2*	*KIAA0931, PHLPPL*	Yes	Occas	Lechner et al.
PI3K-alpha	p100α, PI3Kα, PI3Kalpha	P42336	p.R88Q	*PIK3CA*	N/A	Unkn	Unkn	Wang et al.; Lechner et al.
			p.E542K			Yes	Occas	
			p.H1047L			Yes	Occas	
			p.H510N			Yes	Occas	
PI3K-gamma	p110γ, PI3Kγ, p120-PI3K	P48736	p.S236L	*PIK3CG*	N/A	Yes	Occas	Lechner et al.
PP1R6B	hTIMAP, TIMAP	Q96T49	p.R371Q	*PPP1R16B*	*ANKRD4, KIAA0823*	Yes	Unkn	Agrawal et al.
PTC1	PTC	Q13635	p.T1052M	*PTCH1*	*PTCH*		Occas	Lechner et al.
PTEN	MMAC1	P60484	p.Q245*	*PTEN*	*MMAC1, TEP1*	Yes	Occas	Lechner et al.
Raptor	p150 target of rapamycin (TOR)-scaffold protein	Q8N122	p.R532Q	*RPTOR*	*KIAA1303, RAPTOR*	No	No	Lechner et al.
			p.R1154C			No	Occas	
Rb	pRb, p105-Rb, pp110	P06400	p.R579*	*RB1*	N/A	Yes	Occas	Lechner et al.
R-PTP-delta	N/A	P23468	p.G203R	*PTPRD*	N/A	No	No	Lechner et al.
SCFR	PBT, c-KIT, p145 c-kit, CD117	P10721	p.T488M	*KIT*	*SCFR*	Yes	Occas	Lechner et al.
STAT3	Acute-phase response factor	P40763	p.G388R	*STAT3*	*APRF*	No	No	Lechner et al.
			p.E523K			Yes	Occas	
STK11	LKB1, hLBK1, NY-REN-19	Q15831	p.P324L	*STK11*	*LKB1, PJS*	No	No	Lechner et al.
SUFUH	N/A	Q9UMX1	p.P482L	*SUFU*	*UNQ650/PRO1280*	Yes	Occas	Lechner et al.
Tankyrase-1	TANK1, ARTD5, TNKS-1	O95271	p.M1311I	*TNKS*	*PARP5A, PARPL, TIN1, TINF1, TNKS1*	Yes	Occas	Lechner et al.
TET2	N/A	Q6N021	p.P472L	*TET2*	*KIAA1546, Nbla00191*	No	No	Lechner et al.
			p.Q1542_R1543insQ			No	Occas	
TM7SF3	N/A	Q9NS93	p.N150D	*TM7SF3*	N/A	Yes	Unkn	Agrawal et al.
Trk-B	TrkB, BDNF/NT-3 growth factors receptor	Q16620	p.E371K	*NTRK2*	*TRKB*	Yes	Occas	Lechner et al.
Tuberin	TSC2	P49815	p.A583T	*TSC2*	*TSC4*	Yes	Occas	Lechner et al.
UNC5D	Protein unc-5 homolog 4, Netrin receptor UNC5D	Q6UXZ4	p.H906L	*UNC5D*	*KIAA1777, UNC5H4*	Yes	Unkn	Agrawal et al.
VGFR-2	FLK-1, KDR, CD309	P35968	p.I915T	*KDR*	*FLK1, VEGFR2*	Yes	Occas	Lechner et al.

>24, >24 units of alcohol per week; cons, consumption; N/A, not available; occas, occasional; unkn, unknown.

efforts are expected to prevent HPV viral infection of epithelial cells and its stem cells and likely disrupt the genetic and epigenetic HPV-driven reprograming of normal stem cells and malignant transformation, as also seen in HPV-driven methylation and reprograming of cervix cancer initiating–tumor cells (Leonard et al. 2012).

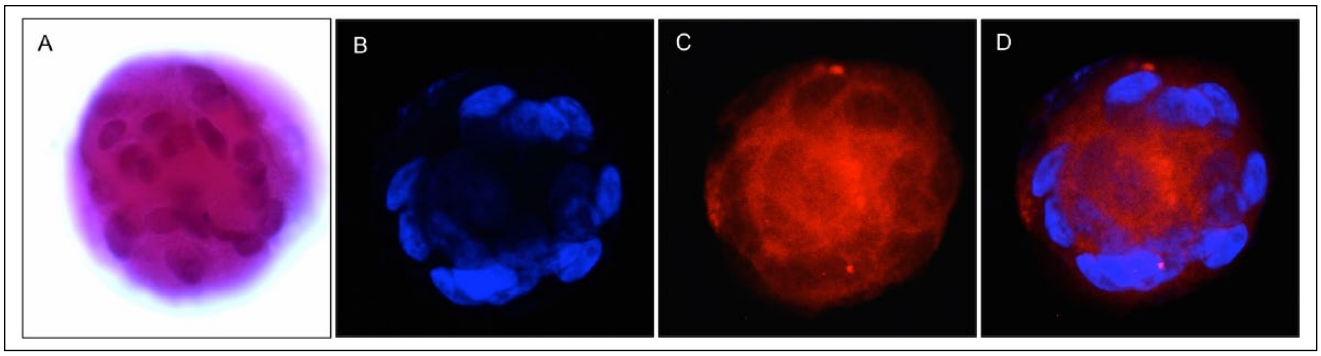

Figure 2. Activation of the phosphoinositide 3-kinase pathway in a head and neck squamous cell carcinoma (HNSCC) cancer stem cell (CSC) in response to human papillomavirus infection. The ability to grow HNSCC CSC spheres in low attachment conditions and the cytospin technology, in which CSC spheres are immobilized and immunostained using an immunofluorescence technique. (**A**) HNSCC CSC accumulation forming an orosphere (hematoxylin and eosin). (**B–D**) An immunofluorescence technique in an orosphere generated from an oropharyngeal cancer cell line. Note pS6 activation (red) in response to human papillomavirus infection. Nuclear counterstain Hoechst 33342 is in blue.

HPV Infection and Cancer Stem Cells

A cancer stem cell (CSC) is defined as a cell within a tumor that can self-renew, fueling tumor growth by generating lineages of cancer cells that compose the majority of transiently amplified-like tumor cells with limited cellular division (Bao et al. 2006; Clarke et al. 2006; Klevebring et al. 2014). CSCs are also known as "tumor-initiating cells," cancer-initiating cells, and "tumorigenic cells" (Clarke et al. 2006).

Recent evidence indicates that CSCs have stem cell–like properties and play a fundamental role in tumor heterogeneity, growth, progression, spreading, and preservation (Lobo et al. 2007). CSCs are also refractory to chemotherapy and radiation. Therefore, CSCs play a major role in treatment failure, tumor recurrence, and metastasis, which are common causes of morbidity and death in the majority of cancer patients, including those with HNSCC (Reya et al. 2001; Bao et al. 2006; Phillips et al. 2006; Rich 2007; Li et al. 2008; Sinha et al. 2013; Zhang et al. 2014).

CSCs were first discovered in solid tumors in 2003 (Al-Hajj et al. 2003) and subsequently in many other tumor types—including leukemia; cancers of the breast, brain, prostate, colon, pancreas, liver, and skin; and, most recently, HNSCC (O'Brien et al. 2009). Side populations of cells with cancer-initiating properties were identified with cell surface markers and dyes, such as CD44, CD24, c-MET, CD133, CD98, and BMI-1, among others (Keysar and Jimeno 2010; Major et al. 2013). These cells have increased clonogenic and tumorigenic potential in HNSCC. HNSCC cells expressing high levels of the CD44 antigen may possess CSC properties, have increased metastatic potential, and be resistant to therapy (Esseghir et al. 2006; Tang et al. 2013; Rietbergen et al. 2014). Interestingly, the CSC enrichment marker CD44 is lower in patients with HPV-positive HNSCC as compared with patients with HPV-negative HNSCC (Rietbergen et al. 2014).

Among the diverse pool of molecular markers used to identify and select CSCs, aldehyde dehydrogenase 1 (ALDH1) is a common marker of pluripotency in solid tumors, including breast, prostate, lung, and pancreas. ALDH1 enzymatic activity identifies normal pluripotent epithelial cells and CSC tumor cells harboring "stemness" potential in HNSCC. ALDH1 enzymatic activity in tumor cells is associated with poor clinical outcomes in breast cancer, ovarian cancer, papillary thyroid carcinoma, and pancreatic adenocarcinoma, among other solid tumors (Tirino et al. 2013; Le et al. 2014).

The use of molecular markers, especially ALDH1 enzymatic activity, allowed the HNSCC CSC isolation and quantification from tissue samples and cell lines. Functional assays—such as 3-dimensional sphere formation in low attachment plates and in vivo transplantation and new tumor formation (of tumor spheres and/or fluorescence-activated cell sorting–isolated CSC)—became the gold standard qualitative assays to study CSC. These qualitative assays may be used, for example, to access autocrine and paracrine stimuli, response to therapy, and altered molecular mechanism in HNSCC CSC (Fig. 2).

To date, the effects of HPV16 on CSCs are poorly understood. One idea in the field was that patients with HPV-positive HNSCC responded more favorably to treatment than did patients with HPV-negative HNSCC because HPV-positive tumors might harbor fewer CSCs. Nevertheless, in vivo limiting cell dilution experiments revealed that CSC frequency is greater in HPV-positive tumors than in HPV-negative tumors (Zhang et al. 2014). Indeed, data from a large cohort of patients indicate that HPV16-positive HNSCC is associated with increased ALDH1 staining in tumor cells (Zhang et al. 2014). Therefore, this study suggested that patients with HPV-positive HNSCC have a better prognosis and respond more favorably to (chemo)radiation therapy than do patients with HPV-negative HNSCC, likely due to CSC phenotype or quality (Almeida and Squarize, unpublished data). In addition, CSCs from HPV-positive and HPV-negative HNSCC cell lines are very resistant to cisplatin therapy (Tang et al. 2013). Moreover, in vivo studies indicated that cells on the bulk of HPV-positive HNSCC may respond better to platinum-based cancer therapy (Spanos et al. 2009; Tang et al. 2013).

HPV-positive HNSCC and Mutations

The majority of mutations found in CSCs are also observed in the bulk tumor cells of HNSCC. Indeed, 83% of mutations are

shared between CSCs and bulk breast cancer cells. These data demonstrate that CSCs and bulk tumor cells share a common genetic background, and there is a dynamic and reversible transition between stemlike and bulk tumor cells in the neoplasm (Chaffer et al. 2011; Gupta et al. 2011; Klevebring et al. 2014). Interestingly, the median mutation allele frequency across patients is 17% (90% of the frequencies were between 3.2% and 50%; Klevebring et al. 2014), and this heterogenicity is observed in HNSCC. In fact, no single genetic mutation or dysregulated pathway is responsible for malignant progression in HNSCC (Agrawal et al. 2011; Stransky et al. 2011; Giudice and Squarize 2013; Cancer Genome Atlas Network 2015).

Understanding the distinct genetic alterations that occur in HNSCC subtypes is vital for utilizing genome-based therapy to personalize treatment. To this end, the global network of associations of the reported mutated proteins found in HPV-positive tumors is shown according to the STRING database (Jensen et al. 2009; Fig. 3). Detailed analysis based on Gene2Networks analysis (Berger et al. 2007) shows that mutations and copy number alterations in the phosphoinositide 3-kinase (PI3K) pathway, receptor tyrosine kinases (RTKs), and genes involved in DNA damage repair are often found in HPV-positive HNSCC (Fig. 4). Furthermore, HPV-positive and HPV-negative HNSCCs have distinct genetic mutations (Agrawal et al. 2011). TP53 mutations are in the majority of HPV-negative tumors but are rarely detected in HPV-positive tumors (Balz et al. 2003; Hafkamp et al. 2003; Westra et al. 2008; Agrawal et al. 2011; Cancer Genome Atlas Network 2015). The presence of wild-type p53 or absence of TP53 mutations on HPV-positive HNSCC has been suggested to positively affect patient outcomes (Sisk et al. 2002), especially considering that nonsynonymous mutations or disruptive mutations are significant predictors of poor patient survival, tumor progression, and response to therapy in HPV-negative HNSCC (Poeta et al. 2007). The selective pressure for TP53 mutations found in HPV-negative tumors may be superseded by the p53 protein degradation by the viral oncoprotein E6 (Scheffner et al. 1990). Consequently, TP53 mutations are found in <25% of HPV-positive HNSCC and are often considered nondisruptive mutations (i.e., mutations that occur outside the DNA-binding motif or do not result in a stop codon and frameshift mutation; Westra et al. 2008; Table 1).

PI3K/mTOR Pathway

The PI3K pathway plays an important role in HPV-positive and HPV-negative HNSCC (Agrawal et al. 2011; Stransky et al. 2011; Lechner et al. 2013; Cancer Genome Atlas Network 2015). Genomic instability is more common in HNSCC tumors with PI3K pathway mutations as compared with tumors without PI3K pathway mutations (reviewed by Giudice and Squarize 2013). PI3K pathway mutations are associated with a greater number of nonsynonymous mutations and mutations in DNA damage/repair genes (Giudice and Squarize 2013).

Notably, a significant relationship exists between HPV status and *PTEN* and *PIK3CA*. More than 55% of HPV-positive tumors have mutated or amplified *PIK3CA* and have inactivated *PTEN* due to gene copy loss or mutation (Lechner et al. 2013; Table 1). Hot spot and missense mutations in *PIK3CA* with nucleotide changes at 3140A>T, 1624G>A, and 1633G>A resulted in amino acid changes on its protein PI3K-α (H1047R/L, E542K, and E545K, respectively), which occur in HPV-positive HNSCC (Agrawal et al. 2011; Lechner et al. 2013; Table 1). These activating mutations increase lipid kinase and AKT signaling activity. Functional studies show that PI3K harboring any of the 3 hot spot mutations induces cell-malignant transformation (Samuels et al. 2004). Additionally, mutations in PI3K-α (H510N) and PI3K-γ (S236L) are found in HPV-positive HNSCC (Lechner et al. 2013).

HPV-positive HNSCC harbors homozygous and heterozygous deletion of *PTEN* or the nonsense mutation with a nucleotide change of 733C>T resulting in a premature stop codon (p.Q245*; Table 1). In addition, >15% of head and neck cancer samples have altered *FBXW7* (Lechner et al. 2013). FBXW7 mutations found in HPV-positive HNSCC include R367*, R479Q, R505C, and R505C/H/L (Table 1), resulting from nucleotide changes 1099C>T, 1436G>A, 1513C>T, and 1514G>A/T, respectively. FBXW7 acts as a tumor suppressor in many types of cancers and targets growth-promoting proteins, including mTOR, Cyclin E, MYC, and NOTCH, for proteasomal degradation (Kox et al. 2010; Schülein et al. 2011). FBXW7 mutations occur in a hot spot known to block the degradation of active NOTCH1 (Baldus et al. 2009). Collectively, these data suggest that NOTCH1 acts as a tumor suppressor in HNSCC. Indeed, numerous missense mutations and deletions on NOTCH1, such as R353H, C1133F, F1292L, R1438H, and M2011R, occur in HPV-positive HNSCC.

Inactivating mutations in STK11, which are associated with metastasis in head and neck cancer (Kline et al. 2011; Lechner et al. 2013), are found in HPV-positive HNSCC due to a nucleotide change of 971C>T at P324L (Table 1). Loss of function of STK11 causes activation of mTORC1 signaling, which sensitizes tumor cells to inhibition of mTOR (Corradetti et al. 2004).

The tumor suppressor gene *TSC1* encodes the protein known as Hamartin or TSC1, which interacts with Tuberin or TSC2 to form the TSC1-TSC2 complex. This complex inhibits signal transduction to the downstream effector mTOR (Corradetti et al. 2004). Mutations in TSC1 and TSC2 at p.H732Y and p.A583T, respectively, and in the mTOR complex at Raptor (p.R532Q) are also found in HPV-positive tumors (Table 1), underscoring the importance of the PI3K/mTOR signaling in HPV-positive HNSCC.

Genomic Instability and DNA Damage

As previously mentioned, dysregulation of the cell cycle induced by E6 and E7 is not sufficient but is necessary for oropharyngeal tumorigenesis (Lechner et al. 2013). Viral oncogenes may contribute to carcinogenesis by inducing cellular genomic instability and aneuploidy through targeting cell cycle checkpoints and antiapoptotic machinery that is responsible for genomic proofreading. Through their role in maintaining the S phase of the cell cycle, E6 and E7 can abrogate cell cycle

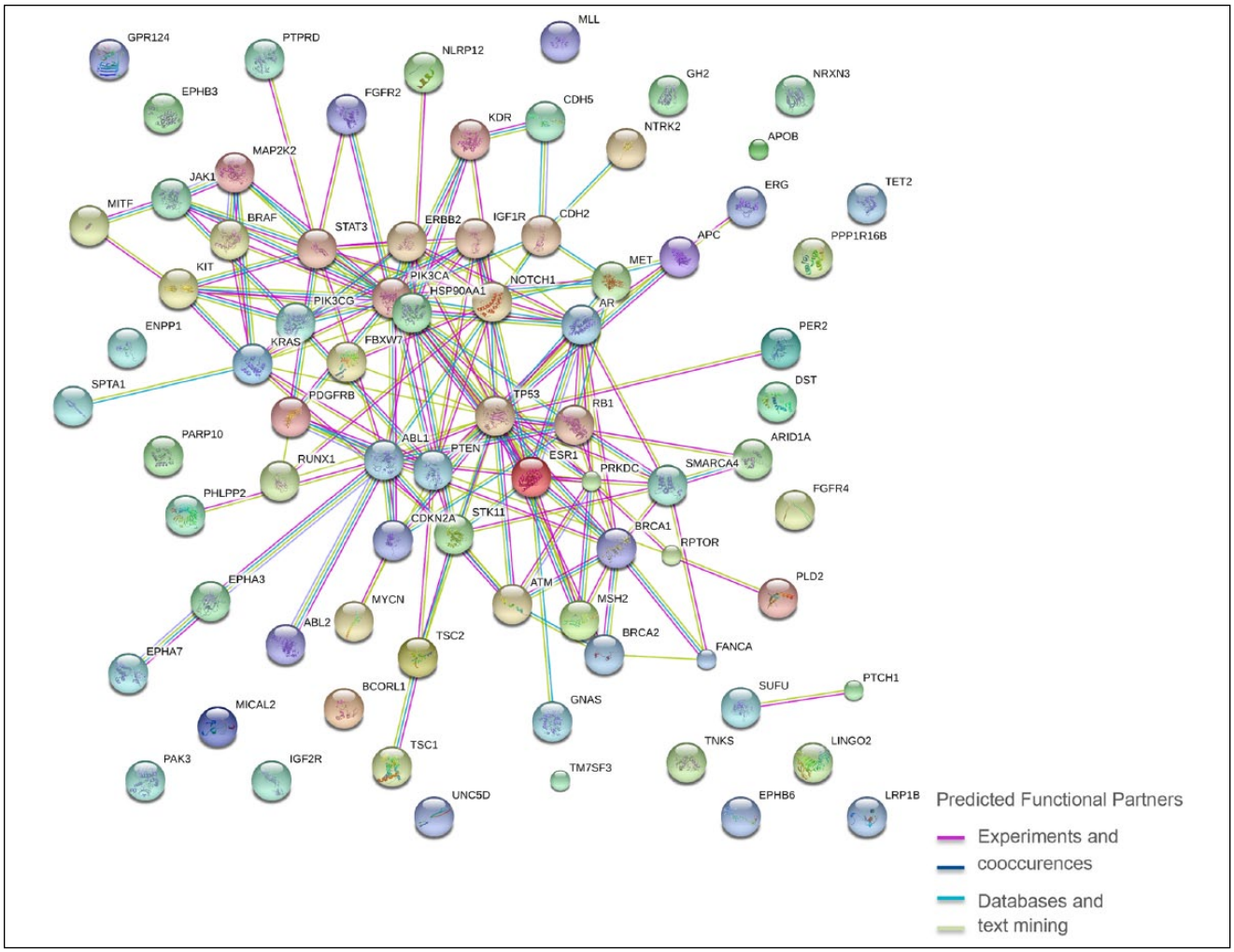

Figure 3. Global view of the molecular network among mutated proteins in human papillomavirus–associated head and neck tumors according to the STRING database. Line colors represent the types of evidence for the association. Network displayed proteins interactions with a confidence score of 0.9. This figure is available in color online at http://jdr.sagepub.com.

checkpoints, resulting in the accumulation of DNA damage, centrosome abnormalities, and mutations. The resulting genomic instability causes cellular transformation and progression to carcinoma (Duensing and Münger 2004; Moody and Laimins 2010).

HPV controls cellular gene expression through histone deacetylases and promotes hyperproliferation through inhibition of retinoblastoma family members. HPV also activates the ATM-ATR pathway, resulting in the accumulation of chromosomal alterations (Moody and Laimins 2010). In HPV-positive HNSCC, many DNA repair molecules are mutated, including ATM, BRAF, BRCA1, BRCA2, FANCA, and RB1 (Table 1).

HPV and Mutated Cell Membrane Receptors

The majority of RTK mutations drive oncogenic transformation, tumor progression, and metastasis. In HPV-positive HNSCC, mutations are found in MET, erbB-2, FGFR-2, FGFR-4, VGFR-2, SCFR (c-KIT), and other RTK receptors (Table 1). In addition, proteins belonging to the ephrin receptor (Eph) subfamily of the protein-tyrosine kinase family are mutated in HPV-positive HNSCC (Table 1).

Ephs are the largest subfamily of RTKs. EphA and EphB are the 2 subclasses of Ephs. Ephs play a critical role during embryogenesis and are rarely expressed in normal adult tissues. However, variable levels of Ephs are found in solid tumors, such as melanoma, breast, prostate, gastric, and colon cancer, resulting in controversy regarding its role in cancer (Pasquale 2010).

Ephrins and Eph receptors are commonly expressed in adult stem cell niches (Genander 2012). Both increased expression and decreased expression of Ephrins and Eph receptors are linked to tumor progression, highlighting the complexity of Eph/ephrin biology. Ephrins and Eph receptors participate in aberrant tumor cell–cell communication and in crosstalk between tumor cells and cells in the microenvironment. Angiogenesis, tumor growth, invasiveness, and metastasis occur in vivo in response to the amount of Eph expression.

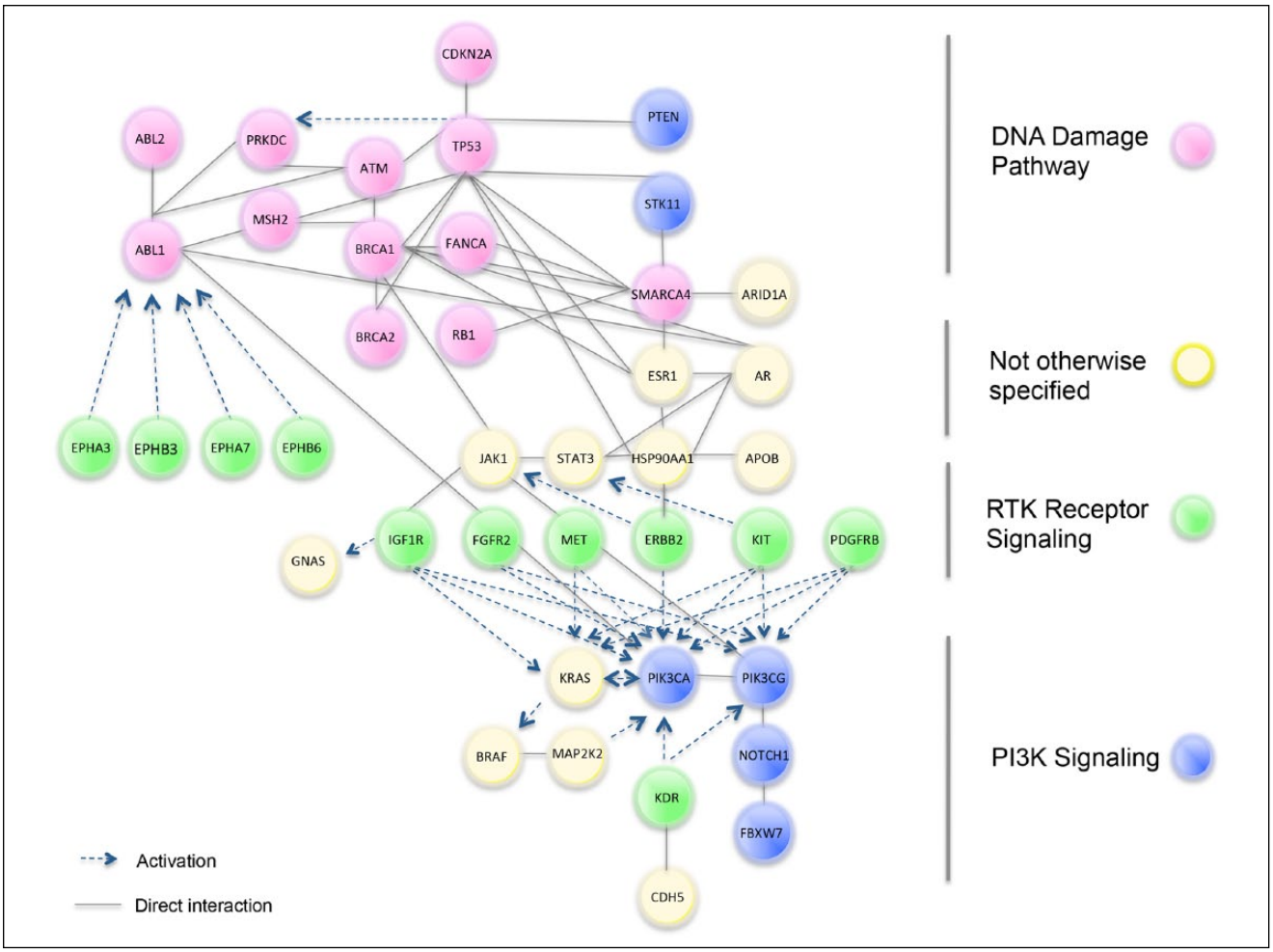

Figure 4. Local pathway view of protein interactions commonly altered in human papillomavirus–positive head and neck cancer. The refined interaction map shows that most mutated proteins belong to the DNA damage (pink), receptor tyrosine kinase receptor family (green), and PI3K pathways (blue). Protein interaction network was made according to Gene2Networks. Lines represent direct protein interaction, and arrows represent protein activation. This figure is available in color online at http://jdr.sagepub.com.

Epigenetic silencing and regulatory events such as promoter hypermethylation and mRNA stability follow upregulation of EphB in advanced colon, prostate, and lung cancers. In addition, decreased ephrin or mutations in Eph are found in skin cancer, colon cancer, glioblastoma, and HNSCC (Pasquale 2010; Giudice and Squarize 2013).

The understanding of the effect of Eph in HNSCC remains limited. Further studies are needed to understand the contribution of Eph and RTKs to HNSCC. It will be interesting to assess Eph and ephrin in large cohorts of HNSCC and correlate their expression with malignant stage and clinical outcome. To advance our understanding of Eph and RTKs in HNSCC, it will also be important to examine the effects of mutation or loss of function of these genes in genetically engineered mouse models that mimic the progression of HNSCC.

HPV Vaccines and HPV Oral Infection

Rather than having to manage cancer, it is ideal if cancer can be prevented, especially if the HPV infection of epithelial cells and stem cells can be avoided. The use of HPV vaccines to prevent HPV-positive HNSCC is not yet recommended by the World Health Organization (WHO), although there are many clinical trials underway (Table 2). The first vaccine against HPV infection was licensed by the US Food and Drug Administration in 2006. This vaccine was approved for females in 2006 and for males in 2011 (Wierzbicka et al. 2014). HPV vaccines are now licensed in many countries worldwide, and the WHO recommends the HPV vaccination to national immunization programs (WHO 2009) to prevent mainly genital HPV infection.

Gardasil (Merck & Co, Whitehouse Station, NJ, USA) and Cervarix (GlaxoSmithKline Biologicals, Brentford, UK) are the 2 commercially available HPV vaccines. Rather than containing HPV DNA, these vaccines use virus-like particles comprising the major capsid protein L1 of the targeted HPV subtypes (Blitzer et al. 2014). Gardasil is a quadrivalent vaccine designed to protect against HPV6, HPV11, HPV16, and HPV18; Cervarix is a bivalent vaccine that protects against HPV16 and HPV18 (Wierzbicka et al. 2014). Therefore, both

Table 2. Registered Clinical Trials of HPV Vaccines and Patients with HPV-positive Head and Neck Squamous Cell Carcinoma.

Product Name	NCT No.	Phase	Status[a]
ADXS11-001 (ADXS-HPV)	NCT02002182	Phase I/II	Active, not recruiting
MAGE-A3 HPV-16 vaccine	NCT00704041; NCT00257738	Phase I	Unknown
NGVL-4a-CRT/E7 (Detox) DNA vaccine	NCT01493154	Phase I	Terminated
Quadrivalent HPV recombinant vaccine (Gardasil)	NCT02382900	Phase III	Recruiting
Synthetic HPV 16 E6 peptide	NCT00019110	Phase I	Completed
VGX-3100	NCT02163057	Phase I/II	Recruiting

HPV, human papillomavirus.
[a]Last verified: June 2015. Sources: ClinicalTrials.gov and World Health Organization International Clinical Trials Registry Platform.

vaccines are used to prevent HPV16 and HPV18 infections and HPV-associated cancer and dysplasia. Notably, Gardasil may also prevent oral papillomatous lesions and genital warts, which are commonly associated with HPV6/11. A 9-valent HPV vaccine is currently in clinical trials. This vaccine, called V503, includes HPV types in the existing quadrivalent vaccine (HPV6/11/16/18) and 5 additional high-risk types (HPV31/33/45/52/58; Tomljenovic et al. 2013).

Additional vaccines against HPV are considered therapeutic vaccines, which boost the immune system to recognize virus-infected cancer cells targeting E6 and E7 oncoproteins (Wierzbicka et al. 2014). This strategy is of particular interest for oropharyngeal HNSCC because the majority of these cancers are HPV16 positive (Gillison et al. 2012). In fact, the "Safety Study of HPV DNA Vaccine to Treat Head and Neck Cancer Patients" is a clinical trial that began in April 2012 to test the safety of pNGVL4a-CRT/E7(Detox), an HPV DNA vaccine (Wierzbicka et al. 2014), and many others are underway (Table 2).

Although safety is always a concern with new vaccination programs, the Global Advisory Committee on Vaccine Safety has not identified safety issues that would alter any of the current recommendations with the HPV vaccines, and it continues to affirm that the benefit-risk profile is favorable (WHO 2009; Schiller and Lowy 2012). Due to the high incidence of HPV-associated cervical cancer worldwide, the aim of the vaccination programs is to reduce genital HPV-related lesions. However, the effects of the HPV vaccine on oral HPV infection and other HPV-positive premalignant and malignant lesions are poorly understood.

By the year 2020, the number of HPV-positive HNSCC diagnosed yearly is expected to exceed the number of invasive cervical cancer diagnoses (Chaturvedi et al. 2011; Gillison et al. 2012). Notably, analysis of oral HPV infection in 7,466 women aged 18 to 25 y revealed a lower prevalence of oral HPV 4 y after HPV16/18 vaccination, with a protective efficacy estimated at 93.3% (Herrero et al. 2013). It was a major discovery in the field, which points toward the use of HPV vaccine to prevent HPV infection of the oral cavity and oropharynx.

Because vaccines are preventative in nature, the first HPV immunization was intended for those aged 9 to 13 y. The efficacy of HPV vaccines has been shown in controlled clinical trials (Schiller and Lowy 2012). Studies in young women have demonstrated consistent, strong, and durable antibody responses to each type of HPV in the vaccine, with seroconversion rates approaching or equal to 100% for each type of HPV vaccine. For women vaccinated with Cervarix, stable detectable responses are maintained for >4 y, with maintenance of plateau levels above what are detected after natural infection observed for up to 8.4 y. Similar results have been reported for Gardasil, with additional evidence showing that revaccination at month 60 results in boosted immune memory of antibody responses (Olsson et al. 2007). Both Gardasil and bivalent Cervarix exhibit excellent safety and immunogenicity profiles, while providing high efficacy against external genital lesions. Although the efficacy of HPV vaccines is high in HPV-negative individuals, interestingly, HPV-positive patients also benefit from immunization, with efficacy rates between 50% and 78% (Schiller and Lowy 2012).

HPV vaccination programs have not been used to prevent HPV infection of the oropharynx and its stem cells. To our knowledge, only 1 study, with women from Costa Rica, analyzed the impact of the HPV vaccine on HPV oral infection, but fortunately, others are underway. This study showed that HPV vaccine has great efficacy in reducing oral HPV infection by 93% in 4 y (Herrero et al. 2013). Additionally, mathematical analysis conducted in Canada suggests that the inclusion of males/boys in the vaccination programs should result in a good cost-effectiveness to prevent oropharyngeal cancer (Graham et al. 2015). It is becoming evident that the HPV vaccines should efficiently protect against oral HPV infection and may prevent HPV-associated HNSCC.

Summary

It became evident that HPV-positive HNSCC has genetic alterations, clinical progression, and therapeutic response distinct from HPV-negative HNSCC. Sequencing studies of HNSCC, including HPV-positive tumors, provided extraordinary characterization and identification of mutations and genetic alterations and further confirmed genotypic heterogeneity in HNSCC patients. A great deal of research is still needed to understand the phenotypic and functional alterations of CSCs, the bulk cells of the tumor, and their interactions with the microenvironment. It is unknown to what extent these genetic alterations contribute to tumor formation, progression, and response to therapy.

Head and neck cancer animal models targeting genetic and environmental changes will be essential to critically test how these various alterations contribute to biological behavior of the tumor and its CSCs. We have only scratched the surface in understanding these heterogeneous tumors and how we can use novel information to improve the prognosis for patients diagnosed with HNSCC. Importantly, understanding how altered molecular pathways in HPV-positive HNSCC influence the behavior and response of CSCs to the tumor microenvironment will provide the foundation for developing novel tailored therapeutic approaches to treat this devastating disease.

Author Contributions

A.N. Pullos, contributed to design and data analysis, drafted the manuscript; R.M. Castilho, C.H. Squarize, contributed to conception, design, and data analysis, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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